Snf1 Kinases with Different β-Subunit Isoforms Play Distinct Roles in Regulating Haploid Invasive Growth

Valmik K. Vyas, Sergei Kuchin, Cristin D. Berkey, and Marian Carlson

Integrated Program in Cellular, Molecular and Biophysical Studies, and Departments of Genetics and Development and Microbiology, Columbia University, New York, New York 10032

Received 4 October 2002/Returned for modification 13 November 2002/Accepted 21 November 2002

The Snf1 protein kinase of Saccharomyces cerevisiae has been shown to have a role in regulating haploid invasive growth in response to glucose depletion. Cells contain three forms of the Snf1 kinase, each with a different β-subunit isoform, either Gal83, Sip1, or Sip2. We present evidence that different Snf1 kinases play distinct roles in two aspects of invasive growth, namely, adherence to the agar substrate and filamentation. The Snf1-Gal83 form of the kinase is required for adherence, whereas either Snf1-Gal83 or Snf1-Sip2 is sufficient for filamentation. Genetic evidence indicates that Snf1-Gal83 affects adherence by antagonizing Nrg1- and Nrg2-mediated repression of the FLO11 flocculin and adhesin gene. In contrast, the mechanism(s) by which Snf1-Gal83 and Snf1-Sip2 affect filamentation is independent of FLO11. Thus, the Snf1 kinase regulates invasive growth by at least two distinct mechanisms.

In fungi such as Candida albicans, Cryptococcus neoformans, and Ustilago maydis, the ability of cells to undergo a dimorphic transition between yeast-like growth and filamentous or hyphal growth is an important determinant of pathogenicity. The budding yeast Saccharomyces cerevisiae also exhibits a dimorphic transition in response to nutrient limitation and provides a convenient genetic system for studying this process. The nature and regulation of the dimorphic transition is determined by ploidy (for reviews, see references 10, 22, and 28). Haploid S. cerevisiae cells initiate filamentous invasive growth upon glucose depletion (7, 37), whereas diploid cells make a transition to pseudohyphal growth in response to nitrogen starvation (12), although filamentous growth can also occur in response to carbon source depletion (8, 21). The distinct form of pseudohyphal growth that occurs in mutants lacking the forkhead transcription factors, which control cell cycle-regulated genes, is nutrient independent (15, 51).

Haploid invasive growth depends on FLO11, a gene encoding a cell surface glycoprotein that functions as a flocculin or adhesin (13, 21, 24, 25, 36). FLO11 has a large and complex promoter that is regulated by the cyclic AMP-dependent protein kinase and mitogen-activated protein kinase pathways (24, 27, 32, 38, 40). The Snf1 kinase, which is required for haploid invasive growth (7), also regulates FLO11 expression in response to glucose depletion by antagonizing Nrg1- and Nrg2-mediated repression of FLO11 (20, 47). One aspect of invasive growth is adherence to the support (36), and Snf1 and the Nrg repressors correspondingly affect the Flo11-dependent adherence of cells to a plastic surface (20). Another aspect of invasive growth is filamentation, which entails cell elongation and a switch from axial to unipolar budding; these morphological changes also require Snf1 (7).

The Snf1 kinase is highly conserved in fungi, plants, and animals (called AMP-activated kinase in mammals), and this family of kinases has broad roles in transcriptional and metabolic regulation in response to stress (for reviews, see references 14 and 19). In the pathogenic yeast C. albicans, Snf1 function is essential for viability (9, 34). In S. cerevisiae, Snf1 is required for many aspects of transcriptional and metabolic adaptation to glucose limitation (5, 11) and has been implicated in other stress responses (1, 43). Besides haploid invasive growth, Snf1 also affects developmental processes such as diploid pseudohyphal growth (20), aging (3, 23), and meiosis and sporulation.

The participation of Snf1 in diverse regulatory responses in S. cerevisiae is facilitated by the existence of multiple forms of the kinase, as is also the case in mammals. S. cerevisiae cells contain three forms, each comprising the catalytic subunit Snf1, the activating subunit Snf4, and one of three β-subunit isoforms, Gal83, Sip1, or Sip2 (17, 49). We will refer to these forms by designating the β subunit, for example, Snf1-Gal83. Although the β subunits exhibit significant functional redundancy, they also have important roles in regulating the specificity of the kinase (3, 42, 45, 49); for example, Gal83 mediates the physical interaction of the kinase with Sip4, a Snf1-dependent transcriptional activator of gluconeogenic genes (45). The β subunits also regulate the subcellular localization of the kinase and presumably its access to different substrates. All of the β subunits are cytoplasmic when cells are grown in abundant glucose; upon glucose depletion, Gal83 directs Snf1 to the nucleus, Sip1 is relocalized apparently to membranes and then to the vacuole, and Sip2 remains cytoplasmic (46). Finally, at least one kinase form is subject to multiple regulatory inputs: Snf1-Gal83 is regulated both by the glucose signaling pathway that inhibits its catalytic activity and by a distinct pathway that controls its localization in response to fermentable carbon sources (46). Thus, the β subunits both confer specificity and provide versatility in the control of different functions of Snf1.

To explore the functions of the Snf1 kinase in regulating invasive growth, we have examined the roles of different Snf1 kinases in adherence to the support and filamentation. We present evidence that Snf1 affects adherence by a pathway
involving the Snf1-Gal83 form, the Nrg repressors, and FLO11. In contrast, both Snf1-Gal83 and Snf1-Sip2 affect filamentation by a FLO11-independent pathway(s). Thus, these studies reveal two distinct mechanisms for regulation of invasive growth by the Snf1 kinase.

MATERIALS AND METHODS

Strains and genetic methods. S. cerevisiae strains used in this work are listed in Table 1. All strains were in the Hansenula polymorpha genetic background and were derived by standard genetic methods (39) from the isogenic strains MY1401, MY1402, and MY1411 of the Sigma2000 series (Microbia, Cambridge, Mass.). gal83Δ::KanMX6: Gal83 is required for invasive growth. Cells were grown in CSM plus 2% glucose to an optical density at 600 nm (OD600) of 0.5 to 1.5, collected, washed, and resuspended to an OD600 of 1 in CSM with 2% glucose.

Microscopy. Cells were grown to stationary phase in YEP plus 2% glucose liquid medium, washed twice with TE buffer, diluted, and spread at low concentration onto CSM with 0.1% or no glucose; 0.1% glucose was as repressive for filamentation as 2% glucose, but overall growth was more closely matched to that occurring on medium with no added glucose. Plates were incubated at 26°C for 16 to 24 h, and microcolonies were viewed using a Nikon Eclipse E800 fluorescence microscope. Images were taken with an Orca100 (Hamamatsu) camera, using Open Lab (Improvision) software, and processed in Adobe Photoshop 5.5.

RESULTS

Gal83 is required for invasive growth. To test the requirement for the different β subunits in invasive growth, we examined isogenic sip1Δ, sip2Δ, and gal83Δ mutants in the S. cerevisiae genetic background. Cell suspensions were spotted on solid CSM containing a low concentration of glucose (0.1%); previously, Cullen and Sprague showed that invasion occurs in response to glucose limitation (7). After incubation for 2 days, the plates were washed. Cells that remained on the plate, having adhered to or penetrated the agar, are described here.
as exhibiting invasive growth (Fig. 1A). The gal83 mutant was markedly defective, as were the snf1 and gal83 sip1 sip2 mutants. The sip1 mutant resembled the wild type, as reported previously (7), as did the sip2 mutant. Although the snf1 and gal83 sip1 sip2 strains grew poorly on 0.1% glucose, the gal83 mutant grew as well as the wild type; hence, there was no correlation between the extent of growth and invasion.

Because the /H9252 subunits exhibit significant functional redundancy, we also constructed double mutants (Fig. 1A). The sip1 sip2 double mutant exhibited invasive growth, indicating that the Gal83 subunit is both necessary and sufficient. The gal83 sip1 mutant was noticeably more proficient in invasive growth than the gal83 single mutant, and the gal83 sip2 sip1 strain was more proficient than the gal83 sip2 strain; these findings suggest that Sip1 inhibits this process, either by a direct mechanism or perhaps indirectly through effects on the metabolic state of the cell. The sip2 mutation slightly reduced invasive-ness of both gal83 and gal83 sip1 strains, suggesting that Sip2 has an auxiliary positive role in invasive growth. A positive effect of Sip2 was similarly apparent in a reg1 gal83 mutant background (see below) (Fig. 1B). This evidence that the Sip2 subunit contributes in some manner to invasive growth was confirmed by subsequent experiments revealing a role in filamentation (see below). Together, these findings indicate that the Snf1-Gal83 kinase has the primary role in invasive growth, but Sip1 and Sip2 also affect this process.

We also assayed invasive growth on YEP plus 2% glucose, which is commonly used for such assays; however, the snf1 and gal83 sip1 sip2 mutants showed a less reproducible defect than on CSM with 0.1% glucose or YEP with 0.1% glucose (data not shown). Invasive growth of the wild type occurs more rapidly on 0.1% glucose than on 2% glucose (Fig. 1B). We suggest that on 0.1% glucose, the Snf1 kinase is critical for an immediate invasive response, whereas during growth on 2% glucose, wild-type and mutant cells differ in their capacity for metabolic adaptation to glucose depletion, which in turn affects the time course of invasion. In previous studies, a snf1:URA3 mutant exhibited a pronounced defect in invasiveness on YEP plus 2% glucose relative to the SNF1 ura3 parent (7), but the magnitude of the defect may have been enhanced by the auxotrophic difference because, in our strains, the ura3 mutation improves invasive growth (data not shown).

To confirm that Snf1-Gal83 is required for invasive growth on YEP plus 2% glucose, we took advantage of our previous finding that a reg1 mutant is much more invasive than the wild type on this medium (20). Reg1 directs protein phosphatase type 1 to inhibit the Snf1 kinase (16, 26, 29, 41, 44), and mutation of REG1 relieves glucose repression of many genes, including FLO11 (20). The effect of reg1 on invasive growth requires the Snf1 kinase (20) and presumably reflects the relief of glucose inhibition of the kinase, because the reg1 mutant was more invasive than wild type on 2% glucose but not on 0.1% glucose (Fig. 1B). The gal83 mutation caused a defect in invasive growth of the reg1 mutant on both YEP plus 2% glucose and CSM with 0.1% glucose; moreover, sip2 further reduced invasion of the reg1 gal83 mutant on both media. These findings exclude the possibility that the effects of gal83 and sip2 are specific to a particular growth medium.

Gal83 affects adherence to plastic. The ability of yeast cells to adhere to a plastic surface is related to their capacity for

FIG. 1. Assay for invasive growth. Cells were grown on solid CSM plus 0.1% glucose (A and B) or on YEP plus 2% glucose containing 2.5% agar (B) for 2 days at 26°C and photographed. Plates were washed and photographed again. The wild-type strain was MCY4565 and mutant strains (listed in Table 1) were also all MATa and carried the same auxotrophic markers (in particular, all are Ura+), so that invasiveness would not be affected by differences in genetic background. On YEP plus 2% glucose, invasive growth of the wild type is easily detectable by 3 days.
invasive growth in that both processes require the Flo11 floc-
culin and adhesin, which is essential for adherence to the
support (13, 36), and both are dependent on Snf1 kinase ac-
tivity (20). We therefore tested the role of Gal83 in adherence
to plastic. Wild-type and gal83 mutant strains were grown to
exponential phase in 2% glucose, resuspended in 0.1% glu-
tose (20). We therefore tested the role of Gal83 in adherence
to polystyrene (36) as described in Materials and Methods.

Evidence that Snf1-Gal83 antagonizes Nrg1 and Nrg2, two
repressors of FLO11. Previous studies showed that one of the
roles of the Snf1 kinase in regulating invasive growth is antag-
onism of the zinc-finger proteins Nrg1 and Nrg2, which repress
FLO11 (20). Both Nrg repressors interact physically with Snf1
(47), and mutation of NRG1 and NRG2 alleviates snf1 mutant
defects in invasive growth, adherence to plastic, and expression
of the STA2 (glucoamylase) promoter (20), which is nearly
identical to that of FLO11 for 3.5 kb. To determine whether
the Snf1-Gal83 form of the kinase is responsible for antago-
nism of Nrg-mediated repression, we examined the genetic
relationships of nrg1 and nrg2 to gal83 with respect to these
phenotypes. First, we assayed invasive growth; in a gal83 mu-
ant, each single nrg mutation had some effect and both nrg
mutations together strongly restored invasive growth (Fig. 3).
The nrg mutations also alleviated the gal83 mutant defect in
adherence to plastic (Fig. 2A). Finally, we assayed expression
of lacZ fused to the STA2 promoter in β subunit mutants
during growth in glucose and after a shift to low (0.05%)
glucose. Derepression of STA2-lacZ was greatly reduced by
gal83 but was not affected by mutation of the other β subunits
(Fig. 4A), and expression was restored in a gal83 nrg1 nrg2
triple mutant (Fig. 4B). These data implicate Snf1-Gal83 in
relief of glucose repression by the Nrg proteins. Taken to-
gether, these genetic findings support the view that the Gal83
subunit mediates the functional interaction of the Snf1 kinase
with Nrg1 and Nrg2.

Sip2 and Gal83 are each sufficient for filamentation. We
next tested the role of different Snf1 kinases in filamentation,
which entails cell elongation and a switch from axial to unipo-
lar budding. Filamentation occurs in response to low glucose,
and a snf1 mutant is defective in both elongation and unipolar
budding (7). We used the assay devised by Cullen and Sprague
(7) to observe morphological changes. Cells were spread onto
CSM lacking glucose and allowed to form microcolonies. Mi-
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poor growth in the absence of glucose. The combinations gal83 sip2 and gal83 sip1 sip2 impaired filamentation in the reg1 mutant background, whereas other combinations had no noticeable effect (Fig. 5B). These findings indicate that either Gal83 or Sip2 is sufficient for filamentation.

The role of Sip2 in filamentation most likely accounts for the observation that mutation of SIP2 in a gal83 strain further diminishes invasive growth (Fig. 1). The following evidence also suggests that Snf1-dependent filamentation contributes to invasive growth. Replacement of the endogenous FLO11 promoter with the S. pombe adh1 promoter was shown to give constitutive, low-level FLO11 expression and to abolish invasion of wild-type cells (31); in our strain background, this replacement similarly abolished invasive growth (Fig. 6B). However, reg1 mutant cells carrying the SpADHp-FLO11 allele filamented in the presence of glucose, unlike wild-type cells (Fig. 6A), and exhibited detectable invasive growth on agar (Fig. 6B). Although it is possible that reg1 upregulates other genes besides FLO11 to affect adherence to agar, these findings are consistent with the idea that Snf1-dependent filamentation contributes to invasiveness.

If Snf1-Gal83 and Snf1-Sip2 affect filamentation through different, redundant pathways, then Snf1-Gal83 could conceivably promote filamentation through the same pathway by which it affects adherence. To address this possibility, we examined the roles of the Nrg repressors and FLO11 in filamentation. The nrg1 nrg2 double mutation did not significantly affect filamentation in an otherwise wild-type genetic background or suppress the filamentation defect of a snf1 mutant on medium containing 0.1% or no glucose (data not shown).

FIG. 4. The gal83 and nrg mutations affect STA2-lacZ expression. Strains with the indicated genotype were transformed with pLCLG-Staf, a centromeric plasmid carrying STA2-lacZ (18). The STA2 promoter is nearly identical to the FLO11 promoter for 3.5 kb, except for two 20- and 64-bp insertions (21). (A) Strains were those shown in Fig. 1. Transformants were grown to mid-log phase in CSM plus 2% glucose lacking leucine to select for the plasmid and were shifted to CSM plus 0.05% glucose for the indicated times. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units, as described previously (47). Values are average activity for four transformants, and standard errors are indicated. (B) Strains were those shown in Fig. 3. Transformants were grown overnight in selective CSM plus 2% glucose, diluted to an OD600 of 0.1 in YEP plus 2% glucose, and grown to an OD600 of 0.5. An aliquot of cells was shifted to YEP plus 0.05% glucose for 180 min. Assays were as described for panel A.

FIG. 5. Microscopic examination of mutants. Cells were spread onto CSM lacking glucose (A) or CSM plus 0.1% glucose (B) and incubated for 16 to 24 h at 26°C to form microcolonies as described in Materials and Methods. Strains were those shown in Fig. 1 and other auxotrophically matched reg1 strains. Bars, 10 μm.
Previous studies showed that diploid filamentation requires *FLO11* (24, 40); in contrast, we found that in haploids, *FLO11* is dispensable, as both *flo11Δ* and *reg1 flo11Δ* mutant cells exhibited filamentous morphology similar to that of the corresponding *FLO11* strains in either 0.1% or no glucose (Fig. 6C). These results are consistent with recent evidence that *flo11* does not affect haploid filamentous morphology in another strain background (8). Thus, the filamentation pathway involving Snf1-Gal83 is distinct from the pathway by which Snf1-Gal83 affects adherence to agar and plastic.

**DISCUSSION**

We have investigated the roles of the Snf1 kinase in regulating haploid invasive growth in response to glucose depletion. We show that different forms of the kinase are required for two different aspects of invasive growth, namely, adherence to the agar substrate and filamentation. The Snf1-Gal83 form of the kinase affects adherence by a mechanism involving the Nrg repressors and *FLO11*. The Snf1-Gal83 and Snf1-Sip2 kinases together affect cell morphology by a mechanism(s) that is independent of Nrg proteins and *FLO11*. Thus, the Snf1 kinase has multiple roles in the complex process of invasive growth (Fig. 7). These findings also provide further evidence that the different β subunits confer specificity to the Snf1 kinase in cellular regulatory responses.

We first showed that the effects of Snf1 on invasive growth are mediated primarily by Snf1-Gal83. Gal83 is both necessary and sufficient for invasive growth, as a *gal83* mutant was defective and a *sip1 sip2* strain was proficient. However, the *sip2* mutation caused a noticeable decrease in invasive growth in a *gal83* mutant background, which we attribute to the defect in filamentation. In contrast, mutation of *SIP1* improved invasive growth in a *gal83* mutant, suggesting an inhibitory role for Sip1.

**FIG. 6.** Agar invasion and filamentation of strains expressing *FLO11* from the *S. pombe adh*’ promoter. (A) Wild-type and *reg1* mutant strains carrying the *SpADHp-FLO11* allele were spread onto CSM containing 0.1% or no glucose and allowed to form microcolonies as described in Materials and Methods. (B) Strains of the indicated genotypes were tested for invasive growth on YEP plus 2% glucose with 2.5% agar at 26°C. Plates were photographed after 10 days. The *REG1 SpADHp-FLO11* strain should have a slight advantage for invasive growth because it carries the *ura3* mutation; nonetheless, the *reg1Δ::URA3 SpADHp-FLO11* strain was more invasive. (C) Strains of the indicated genotypes were examined as described for panel A. Bars, 10 μm.

**FIG. 7.** Model for roles of the Snf1 kinase in invasive growth. The Snf1-Gal83 form of the kinase regulates *FLO11* and possibly other Nrg-repressed genes that affect adhesion. Snf1 may also affect *FLO11* expression by other mechanisms. Snf1-Gal83 and Snf1-Sip2 affect filamentation by a pathway(s) that does not involve *FLO11* or Nrg repressors. (A) These two kinase forms may function redundantly in the same pathway. (B) Alternatively, the two kinases may function in two distinct pathways, either of which suffices for filamentation. Moreover, the role of Snf1 in filamentation may involve multiple targets. Snf1-Sip1 also has an inhibitory effect on invasive growth, but the mechanism is not yet understood.
Snf1-Sip1 may directly inhibit some function that promotes invasive growth or may act indirectly through effects on the metabolic state of the cell. We think it unlikely that loss of Sip1 simply affects the distribution of the Snf1 catalytic subunit among the different isoforms because Sip1 is not an abundant β subunit and because loss of Sip1 has an effect in a gal83 sip2 mutant.

Genetic evidence indicates that the effects of Snf1-Gal83 on adherence occur, at least in part, by inhibition of the Nrg1 and Nrg2 repressors. These repressors may in fact be the major targets, because adherence to both agar and plastic substrates was effectively restored in a gal83 mutant by the double nrg1 nrg2 mutation. Snf1 interacts physically with Nrg1 and Nrg2 (47), and one possibility is that Gal83 interacts directly with these repressors, as it does with the activator Sip4 (45). We were unable to detect two-hybrid interaction of Gal83 with Nrg1 or Nrg2 in several different assays (V. K. Vyas and C. D. Berkey, unpublished results), but these negative results do not exclude the possibility that Gal83 contributes to the Snf1-Nrg interaction. Alternatively, Gal83 could simply facilitate the interaction between Snf1 and these repressors by virtue of its role in the nuclear localization and nuclear function of the kinase (46). Gal83 is the β subunit that is primarily responsible for the nuclear localization of Snf1 upon glucose depletion (46), and Nrg1 is a nuclear protein (47).

We present evidence that one of the functional targets of the Snf1-Gal83/Nrg pathway is FLO11, which is essential for invasive growth and adherence to plastic (21, 24, 25, 36). This pathway may also regulate other genes needed for invasive growth, as only a few Nrg1- and Nrg2-repressed genes besides FLO11 have been identified (20, 33, 47, 50). It is noteworthy that an ortholog of the Nrg proteins in the fungal pathogen C. albicans has been shown to repress filamentous growth and expression of the hypha-specific adhesin genes HWPI, ALS3, and ALS8 (4, 30).

We also examined the role of the Snf1 kinase in promoting filamentation and found that either the Snf1-Gal83 or Snf1-Sip2 form is sufficient. The simple model is that the Snf1 kinase affects filamentation by a pathway in which both Snf1-Gal83 and Snf1-Sip2 can participate, such that the presence of either one alone suffices for function of the pathway (Fig. 7A). Although Gal83 becomes enriched in the nucleus in response to glucose depletion while Sip2 remains predominantly cytosolic (46), their different localizations do not preclude performance of an identical function. Alternatively, the Snf1 kinase could promote filamentation by two redundant pathways, one involving Snf1-Gal83 and the other Snf1-Sip2; however, Snf1-Gal83 must act through a pathway different from that by which it regulates adherence (Fig. 7B). The function(s) of the Snf1 kinase in filamentation could potentially involve transcriptional control or regulation of metabolic enzymes or other cellular components. Very recent work indicates that one function of Snf1 is to promote the disappearance of the axial budding determinant Ax11 in response to glucose limitation (8).

This study thus identifies at least two distinct signaling mechanisms by which the Snf1 kinase contributes to control of two aspects of invasive growth. It is likely that Snf1 participates in yet other regulatory interactions besides those represented in Fig. 7; for example, Snf1-Gal83 may relieve Nrg-mediated repression of other genes besides FLO11, as mentioned above. The involvement of both Snf1-Gal83 and Snf1-Sip2 also raises the possibility that adherence and filamentation are differentially regulated by nutrient signals. A glucose signaling pathway inhibits the Snf1 catalytic activity, but a distinct pathway regulates the nucleocytoplasmic distribution of Snf1-Gal83 in response to fermentable carbon sources, whereas Snf1-Sip2 is constitutively cytoplasmic (46). Further studies are needed to resolve the various roles of Snf1 in the very complex regulatory network that controls invasive growth.

ACKNOWLEDGMENTS

We thank A. Amon, A. Wach, R. Rothstein, and R. Reid for plasmids.

This work was supported by NIH grant GM34095 to M.C.

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